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3100 Ricketts Point Road
Aberdeen Proving Ground, MD 21010-5400

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A Gas Chromatographic–Mass Spectrometric Approach to Examining Stereoselective Interaction of Human Plasma Proteins with Soman*

David T. Yeung[1,2], J. Richard Smith[3], Richard E. Sweeney[4], David E. Lenz[1], and Douglas M. Cerasoli[1,†]

[1]Physiology and Immunology Branch, Research Division, U.S. Army Medical Research Institute of Chemical Defense, 3100 Ricketts Point Road, Aberdeen Proving Ground, Maryland 21010-5400; [2]Department of Pharmacology and Experimental Therapeutics, University of Maryland at Baltimore, 655 W. Baltimore Street, Baltimore, Maryland 21201; [3]Medical Diagnostic and Chemical Branch, Analytical Toxicology Division, U.S. Army Medical Research Institute of Chemical Defense, 3100 Ricketts Point Road, Aberdeen Proving Ground, Maryland 21010-5400; and [4]RESECO Research Engineering Consultants, P.O. Box 554, Nottingham, Pennsylvania 19362

Abstract

The organophosphorus (OP) nerve agent soman (GD) contains two chiral centers (a carbon and a phosphorus atom), resulting in four stereoisomers (C+P+, C–P+, C+P–, and C–P–); the P– isomers exhibit a mammalian toxicity that is ~1000-fold greater than that of the P+ isomers. The capacity to assess the binding or hydrolysis of each of the four stereoisomers is an important tool in the development of enzymes with the potential to protect against GD intoxication. Using a gas chromatography–mass spectrometry-based approach, we have examined the capacity of plasma-derived human serum albumin, plasma-purified human butyrylcholinesterase, goat milk-derived recombinant human butyrylcholinesterase, and recombinant human paraoxonase 1 to interact with each of the four stereoisomers of GD in vitro at pH 7.4 and 25°C. Under these experimental conditions, the butyrylcholinesterase samples were found to bind GD with a relative preference for the more toxic stereoisomers (C–P– > C+P– > C–P+ > C+P+), while human serum albumin and paraoxonase 1 interacted with GD with a relative preference for the less toxic isomers (C–P+/C+P+ > C+P–/C–P–). The results indicate that these human proteins exhibit distinct stereoselective interactions with GD. The approach described presents a means to rapidly assess substrate stereospecificity, supporting future efforts to develop more effective OP bioscavenger proteins.

Introduction

Organophosphorus anticholinesterases (OPs) are among the most toxic substances identified (1–8). Originally, OPs were developed for use as insecticides (3,6,7), but their extreme toxicity toward higher vertebrates because of their ability to inhibit acetylcholinesterase (AChE) has led to their adoption as weapons of warfare (7–9). The OPs most commonly utilized as chemical weapons (usually referred to as nerve agents) are tabun (ethyl dimethylamidocyanophosphate, or GA), sarin (isopropyl methylfluorophosphonate, or GB), soman (pinacolyl methylfluorophosphonate, or GD), cyclosarin (methyl cyclohexylfluorophosphonate, or GF), VX [*O*-ethyl *S*-(2-diisopropylaminoethyl) methylthiophosphonate], and VR [*O*-isobutyl *S*-(2-diethylaminoethyl)methyl thiophosphonate, or Russian VX] (7,10). Although documented cases of nerve agent usage are rare, GB was used against civilian populations in Japan by the Aum Shinrikyo terrorist group, and GA and GB were used by the regime of Saddam Hussein in Iraq (11–14). Thus, nerve agents are known threats to civilian populations as well as military forces. Similarly, many commonly used OP pesticides and chemical manufacturing by-products are also anticholinesterases that are regarded as potentially toxic to military and civilian populations (15). It is therefore very important that more effective medical countermeasures against these toxicants be developed.

Although pharmacological treatments such as anticonvulsants, acetylcholine receptor antagonists, and oximes therapies are in place to counteract the immediate effects of OP nerve agent intoxication (2–4,16), these therapies do not directly detoxify OPs in vivo and usually result in behavioral incapacitation. To overcome these disadvantages, the concept of using

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† Author to whom correspondence should be addressed. U.S. Army Medical Research Institute of Chemical Defense, 3100 Ricketts Point Road, Aberdeen Proving Ground, MD 21010-5400. E-mail: douglas.cerasoli@us.army.mil.

a biological scavenger has emerged as a new approach to reduce the in vivo toxicity of OP nerve agents (17). Bioscavengers fall into two broad categories: stoichiometric [such as human serum albumin (HSA) and human butyrylcholinesterase (HuBuChE)], that is, proteins that detoxify a poison by binding it in some fixed molecular ratio, and catalytic [such as human serum paraoxonase-1 (HuPON1)], that is, proteins that can cause the breakdown of a molecule of a poison, regenerate, and then repeat the process until all of the poison molecules have been destroyed (18,19). To be an improvement over current treatment, a bioscavenger should have minimal or no behavioral or physiological side effects, should provide protection against exposure to as much as five median lethal doses of one or more nerve agents, and should reduce or eliminate any behavioral or physiological side effects normally associated with the currently fielded therapy. Bioscavenger proteins, either as single enzymes or in combinations of multiple enzymes, with a capacity to interact with and detoxify all known nerve agents must be defined. Finally, given the great disparity in the toxicity of different stereoisomers of the nerve agents (20), it would be highly advantageous if the potential bioscavengers were specific for the more toxic isomer(s).

In the present work, we studied the relative selectivity of potential stoichiometric [HSA, HuBuChE purified from human plasma, and goat milk-derived recombinant BuChE (rBuChE)] and catalytic (recombinant HuPON1) bioscavenger proteins for the stereoisomers of GD. The experiments were performed utilizing racemic mixtures of the nerve agent at various concentrations using a chiral gas chromatography–mass spectrometry (GC–MS) approach. This approach allowed for the simultaneous monitoring of the interaction of all four stereoisomers with the scavenger proteins, elucidating the relative stereoselectivity of the different bioscavenger candidates.

Experimental Procedures

Materials

Racemic GD (96.7% pure) was obtained from the United States Army Research Development and Engineering Command (Aberdeen Proving Ground, MD). Immediately before use, the GD was diluted to the indicated concentrations in 50mM glycine/NaOH buffer with 10mM $CaCl_2$ (pH 7.4).

Ethyl acetate (GC-grade) was obtained from EM Science, (Cherry Hill, NJ) and dried over molecular sieve (Fisher Scientific, Fairlawn, NJ) before use. Diisopropylfluorophosphate (DFP), sodium fluoride (NaF), and HSA were purchased from Sigma-Aldrich (St. Louis, MO).

Incubation of GD with PON1

Hydrolysis of GD by recombinant HuPON1 was determined at room temperature as detailed previously with minor variations (21–25). Specifically, GD hydrolysis experiments were carried out using 1.50 mL of supernatant from cells transfected with either the wild-type HuPON1 gene or with empty vector (utilized as a negative control), produced as described (26). Supernatants were incubated with the varying concentrations of GD. Total reaction volume was 3.0 mL. At selected time intervals, 400-µL aliquots were removed and inactivated through extraction with an equal volume of ethyl acetate. The organic layer (containing unhydrolyzed GD) was then removed and dried over molecular sieve. A 50-µL sample of this dried sample was collected and spiked with an internal standard (50µM DFP) before analysis by GC–MS (25). The quantity of GD in each sample was determined by comparison to both the DFP internal standard and a standard GD calibration curve. Calibration curves were obtained by using GD at five different concentrations, spiked with 50µM DFP in ethyl acetate as an internal standard. Kinetic parameters of GD hydrolysis were determined using at least eight different initial substrate concentrations that ranged from 0.2 to 3.0mM. For each concentration of GD, a negative control sample using supernatant from cells transfected with empty vector was analyzed to account for loss of GD due to spontaneous hydrolysis or other non-enzymatically driven processes.

Incubation of GD with HSA or HuBuChE proteins

To analyze the binding of GD to stoichiometric scavengers, 125µM of HSA, 50µM goat milk-derived recombinant human BuChE (kindly provided by Nexia Biotechnologies, now known as PharmAthene, Annapolis, MD), or 30µM human plasma-derived BuChE (Baxter HealthCare, Westlake Village, CA) in 100mM phosphate buffer (pH 7.4) was incubated with varying concentrations of GD (250 µL total reaction volume) for either 30 min (for BuChE samples) or 120 min (for HSA) at room temperature. Following incubation, 250 µL ethyl acetate containing 50µM DFP as an internal standard was added to the samples to extract unbound GD in solution as described previously. The organic layer was dried over molecular sieve and removed for subsequent GC–MS analysis.

Excess fluoride/racemization control experiments

To determine whether racemization of GD occurs at an appreciable rate under the experimental conditions used for the evaluation of catalytic bioscavengers, three independent control experiments were performed under the same conditions as those used to determine the calibration curve. First, 1.0mM racemic GD was incubated with an empty vector control in the presence of excess fluoride ions (2.0mM NaF). Second, semi-purified individual stereoisomers [prepared in ethyl acetate by the TNO Prins Maurits Laboratory (Rijswijk, The Netherlands) (20)] were also incubated with excess fluoride ions using NaF dissolved in saline. Finally, wild-type HuPON1 was reacted with 2mM GD as described previously, but in the presence of 1mM NaF. Each of these control samples was then analyzed by GC–MS analysis.

GC–MS analysis

GC separation of the GD stereoisomers was performed using a modification of a previously developed method (21). An Agilent 6890 GC (Palo Alto, CA) was fitted with a 2.0-m × 0.25-mm internal diameter Chiraldex γ-cyclodextrin trifluoroacetyl column with 0.125-µm film thickness (Advanced Separation Technologies, Whippany, NJ). A 2.5-m × 0.25-mm internal diameter cyano/phenyl/methyl deactivated fused silica retention

gap (Chrompack, Raritan, NJ) was installed at the injection end of the GC and connected to the analytical column using a Chrompack deactivated Quick-Seal glass connector. Helium was used as the carrier gas at a linear velocity of 45 cm/s. The oven temperature was held initially at 80°C for 14 min, programmed from 80 to 90°C at 5°C/min, and held at 90°C for 3 min. Split injections of 1-µL volume were made using an Agilent 7683 autosampler. The injection port temperature was 210°C and the split ratio approximately 1:100. The GC was interfaced to an Agilent 5973 MS with an electron impact ion source. The MS operating conditions were as follows: ion source pressure approximately 1.0×10^{-5} torr; source temperature, 230°C; electron energy, 70 eV; electron multiplier voltage +200 V relative to the autotune setting; and transfer line temperature, 230°C. The MS was operated using selected ion monitoring (SIM). Four ions (*m/z* 69, 82, 99, and 126) were monitored for the GD stereoisomers at a dwell time of 50 m/s for each ion, resulting in a scan rate of 3.77 cycles/s. Three ions (*m/z* 69, 101, and 127) were monitored for DFP. A dwell time of 50 ms for each ion resulted in a scan rate of 5 cycles/s. The *m/z* 126 and 127 ions were used for quantitation of GD and DFP, respectively. To determine the elution/retention time profiles of the four GD stereoisomers, samples of individual stereoisomers were run under the same conditions as those used to determine the calibration curve.

Results and Discussion

GD, a highly toxic AChE inhibitor, contains two chiral centers at the phosphorus atom and at an alkyl side-chain carbon atom. Thus, four stereoisomers (C+P+, C+P–, C–P+, and C–P–) of GD exist (20,26–30). Both of the P– isomers (C+P– and C–P–) are much more toxic in vivo and more readily inhibit AChE in vitro than the P+ isomers (20,26,28,31). Upon binding to AChE, GD covalently reacts with the active site serine through the chiral phosphorus atom, inactivating the enzyme (8,22,32,33).

Using a Chiraldex γ-cyclodextrin trifluoroacetyl column (21), all four stereoisomers of GD and an internal standard (DFP, present during the GC–MS analysis but not during the interaction of GD with proteins) can be quantitatively separated. The elution order of individual GD stereoisomers from a racemic sample was determined by examining the retention times of individual purified stereoisomers alone (data not shown). The relative elution order was C–P–, C–P+, C+P–, and then C+P+ at approximately 11.8, 12.6, 13.0, and 13.4 min, respectively, after injection. When racemic samples of GD were separated, all four stereoisomers were detected in approximately equal ratios. The DFP standard eluted after all four GD stereoisomers, at roughly 17.3 min post-injection. The distinct separation of peaks in the elution profile allowed for the simultaneous determination of the fate of all four GD stereoisomers in the presence of potential bioscavenger proteins [Figures 1 and 2 (20,32,34)].

The candidate bioscavenger proteins studied included HSA, human BuChE (plasma derived and recombinant), and recombinant HuPON1 (23,35–43). HSA is the most abundant protein in serum, while BuChE is the protein most closely related to AChE, the toxic target of OP nerve agents; both are commonly referred to as stoichiometric bioscavengers because they possess the capacity to bind one molecule of nerve agent per molecule of protein scavenger (36,44,45). As a result, the analytical approach used here to study stoichiometric scavengers absolutely required the concentration of the protein and the GD to be at or near an equi-molar ratio. Excessive amounts of GD would mask any stereoselective preferences that the protein may display once all the available binding sites are saturated, and low amounts of GD would result in either insufficient signal or failure to detect stereoselectivity due to binding of all the available GD. Likewise, a sufficient incubation time is critical to ensure adequate GD-enzyme binding has occurred. Interestingly, though all three proteins (HSA,

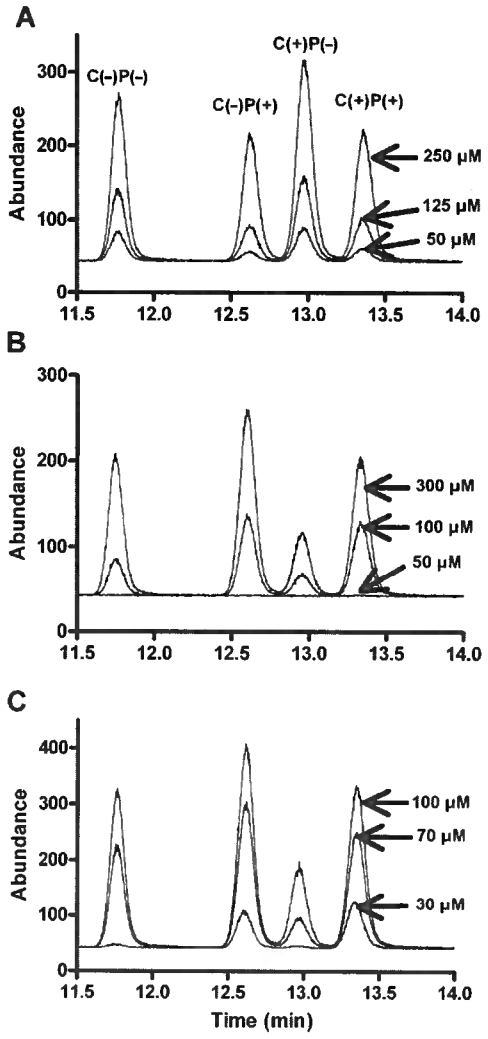

Figure 1. GC–MS reconstructed ion chromatograms of the *m/z* 126 ion of GD at various input concentrations are shown after incubation with human serum albumin (A), goat milk-derived recombinant human butyrylcholinesterase (B), and plasma-derived human butyrylcholinesterase (C). The various GD stereoisomers were eluted in the order shown. Incubation and analysis conditions are as indicated in Experimental procedures.

rBuChE, and HuBuChE) are stoichiometric binders of OPs and can bind all four stereoisomers of GD, Figure 1A shows that HSA preferentially bound the less toxic C±P+ enantiomers, because it is these isomers that display selectively lower peak areas after incubation with protein. Conversely, both HuBuChE and rBuChE preferentially bound the more toxic C±P– isomers (Figure 1B and 1C); a comparison with the relative distribution of the four isomers shown in the zero time-point (before interaction with protein) of Figure 2 is informative in this regard. It is also apparent from the results that the proportion of unbound GD stereoisomers increased as the concentration of GD used increased, which clearly indicated that under the experimental conditions used, all the available binding sites for GD on the proteins were saturated.

Although stoichiometric bioscavengers such as HSA and BuChE represent potential anti-OP therapeutics, they are not ideal. The molecular mass ratio of protein scavenger to nerve agent is approximately 500:1, meaning that a high concentration of scavenger protein in circulation would be required to protect against even a relatively low exposure to nerve agent (46). Subsequently, inactivation of OP nerve agents by catalytically active enzymes has been suggested as a possible alternative approach. In this regard, HuPON1 has promise as an in vivo catalytic bioscavenger of OP nerve agents.

Upon co-incubation with GD, HuPON1 catalyzes the hydrolytic cleavage of the phosphorus-fluorine (P-F) bond in the OP to form P-OH, rendering GD non-toxic (31,32). In the presence of a racemic mixture of GD, the catalyzed reaction is analogous to simultaneously deriving the kinetic constants for the hydrolysis of four competitive substrates. To solve the kinetic parameters, we utilized a previously published model of GD-HuPON1 interaction (25) to determine K_M values of HuPON1 for each of the four stereoisomers of GD; the values ranged from 0.27 to 0.91mM in the following order: C–P– > C+P– > C–P+ > C+P+. The k_{cat} values for the hydrolysis of each stereoisomer were also determined, where the k_{cat} values range from 501 to 1030 min where C+P+ > C–P+ > C+P– > C–P– (Table I). The catalytic efficiency values derived from the model ranged from 625 to 4130mM/min with C+P+ > C–P+ > C+P– > C–P–. The mean K_M, k_{cat}, and k_{cat}/K_M for all four GD stereoisomers in aggregate are 0.62mM, 669 min, and 1739mM/min, which is in reasonable agreement with previously reported values obtained using a racemic mixture of GD and plasma derived HuPON1 in a different assay of enzymatic activity (47). The kinetics of HuPON1-mediated GD (2mM) hydrolysis, determined in the presence of added NaF (1mM), were indistinguishable from those measured in the absence of NaF (data not shown); these results indicate that under the experimental conditions used here, fluoride ions liberated from hydrolyzed GD do not enhance racemization of GD or influence the stereospecificity of HuPON1 mediated GD hydrolysis.

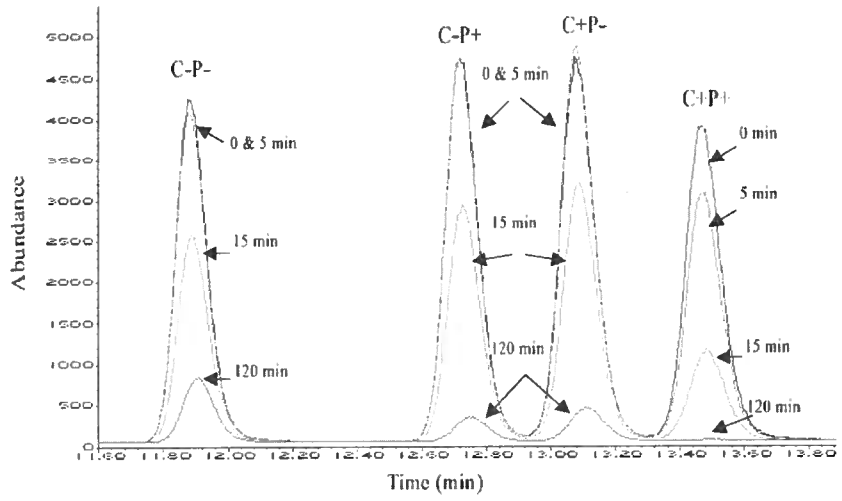

Figure 2. Overlay of reconstructed ion chromatograms (*m/z* 126) of GD hydrolysis by HuPON1 (21). Typical ion chromatograms indicating the relative abundance of the four GD stereoisomers (0.75mM racemic GD) after different incubation periods (i.e., 0, 5.0, 15.0, and 120 min, as indicated) with wild-type HuPON1 enzyme. The various GD stereoisomers were eluted in the order shown.

Table I. Kinetic Parameters for the Enzymatic Hydrolysis of the Various GD Stereoisomers by Recombinant HuPON1*

GD isomer	K_M (mM)	k_{cat} (min^{-1})	k_{cat}/K_M (mM^{-1} min^{-1})
C–P–	0.91 ± 0.34	501 ± 45	625 ± 241
C–P+	0.58 ± 0.23	593 ± 54	1160 ± 469
C+P–	0.71 ± 0.49	553 ± 163	1040 ± 465
C+P+	0.27 ± 0.08	1030 ± 94	4130 ± 1090

* HuPON1 catalyzed GD hydrolysis was assayed in the presence of at least 1.0mM $CaCl_2$ as described in Experimental procedures. Kinetic results presented for each isomer were determined from at least eight independent kinetic experiments ($n \geq 8$) (25).

The study presented here addresses the advancement of an analytical method capable of distinguishing between all four stereoisomers of a racemic mixture of GD simultaneously to determine the preferential decrease of specific isomers over time with catalytic enzymes or after binding to stoichiometic scavenger proteins. Using this technique, the work described has characterized the relative order of preference that potential bioscavengers display for the four stereoisomers of GD. The abundant human plasma protein HSA was found to preferentially bind the less toxic isomers of GD, supporting the suggestion that this protein could serve as a biomarker of exposure to OPs, but may be a poor choice for use as a bioscavenging protein (36). BuChE was found to preferentially bind the more toxic stereoisomers of GD, supporting the potential use of this enzyme as an in vivo scavenger of OPs (40–42,44–46). Furthermore, this study has shown that rBuChE from goat milk displayed the same stereoselective preference for GD as HuBuChE purified from human plasma,

indicating that the stereoselectivity of BuChE is not determined by the source of the protein. This approach has also indicated that although HuPON1 displays selectivity for the less toxic stereoisomers of GD, this selectivity is relatively modest compared to other catalytic anti-GD enzymes that have been characterized (47). The analytical approach described here will likely be of use in determining the stereoselectivity of interaction between other candidate scavenger proteins and GD or related OP nerve agents. As such, it can serve as a screening tool to assess the relative utility of novel scavenger proteins.

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